Protocol for Revolune Illuminate-P4-R, manual staining

Instructions for Use

Product description

This kit is designed to perform single or multiplex immunofluorescence stainings of FFPE (Formalin-Fixed Paraffin-Embedded) samples.

The user can select up to four different rabbit IgG primary antibodies and need to pre-label them with 4 different Revolune Connectors (488, 555, 647 & 750). The staining protocol will be performed manually. The reagents are sufficient to stain 10 slides.

Product contents

Name	Quantity	Use
Revolune Amplifier A (4-plex)	1 mL	First amplification
Revolune Amplifier B (4-plex)	1 mL	First amplification
Revolune Amplifier C (4-plex)	1 mL	Second amplification and signal generation
Revolune Amplifier D (4-plex)	1 mL	Second amplification and signal generation
Revolune Connector 488 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolune Connector 555 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolune Connector 647 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolune Connector 750 (anti-rabbit IgG)	15 µL	Primary Ab labelling

Storage: All contents must be stored refrigerated (2-8°C). Revolune Amplifier C and D are light sensitive and the reagents should generally be stored in the dark.

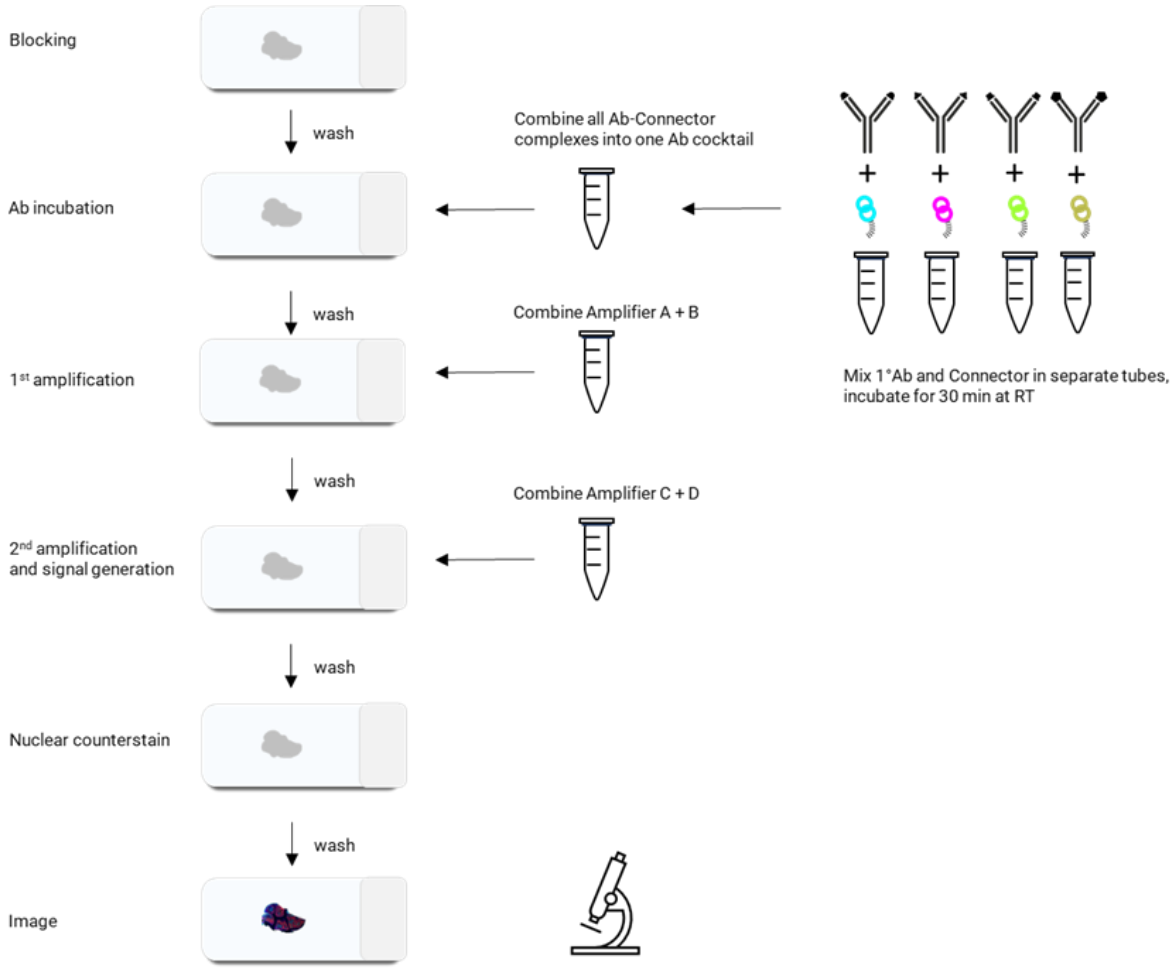
Reagents are stable for 3 months. Use within 4 weeks after opening.

Necessary reagents and consumables (not provided)

- 1x Phosphate Buffered Saline (PBS, pH 7.4)
- 1x PBS-T (1x PBS, pH 7.4, 0.1% Tween) wash buffer
- Antibody diluent (1% BSA in PBS-T, pH 7.4)
- Dewax solution
- Epitope Retrieval Buffer (e.g. citrate buffer, pH 6.0 or Tris-EDTA, pH 9.0)
- Primary antibody (host species, isotype: rabbit, IgG)
- Staining jars (for washing steps), 200-300 mL
- Hydrophobic barrier PAP pen
- Ethanol (Histology-grade, >98%)

- DAPI nuclear stain (working concentration 1 µg/mL)
- ProLong Gold Antifade mounting reagent (Invitrogen, cat.no. P36930). ***Note: Other mounting media may lead to rapid degradation of the signal***
- Standard lab supplies (pipettes/ tips, Eppendorf tubes)
- Glass coverslips
- Optional: Lab orbital shaker

Overview of workflow



Protocol

Part 1: Antibody preparation

In this step, the primary antibody (1° Ab) needs to be pre-labelled with Revolune Connectors. For calculation of the correct volumes, three variables (A,B,C) are needed:

- **A:** Stock concentration of 1° Ab [mg/mL]
- **B:** Working concentration of 1° Ab [µg/mL]
- **C:** Working solution volume [mL]

The stock concentration is typically provided by the antibody manufacturer.

The optimal working concentration of 1°Ab needs to be determined by the user. A good starting point is a concentration that works well for chromogenic IHC (e.g. DAB). Typical working concentrations are in the range of 0.2 µg/mL – 2 µg/mL.

The working solution volume [mL] depends on the number of slides to be stained. For manual staining, ca. 180 µL Ab working solution is used per slide. This can be adjusted based on tissue size.

Based on the three variables A, B & C, the volume of 1° Ab (**D**) and volume of Revolune Connector (**E**) (5 µL per 1 µg 1°Ab) are calculated as follows:

$$D = (B \div A) \times C$$

$$E = B \times C \times 5 \frac{\mu L}{\mu g}$$

Calculation example: A = 1 mg/mL, B = 1 µg/mL, C = 2 mL

$$D = 1 \frac{\mu g}{mL} \div 1 \frac{mg}{mL} \times 2\, mL = 2\, \mu L$$

$$E = 1 \frac{\mu g}{mL} \times 2\, mL \times 5 \frac{\mu L}{\mu g} = 10\, \mu L$$

Each 1° Ab needs to be labelled with a different Revolune Connector. **It is essential to use the correct amount of Revolune Connectors to saturate all binding sites on the 1° Ab to avoid cross-talk in a multiplex experiment**. For example, 1° Ab against target 1 is labelled with Revolune Connector 488 (488 channel), 1° Ab against target 2 with Revolune Connector 555 (555 channel), etc.

The labelling should be performed in separate reaction tubes. Please start with 10 µL PBS and add suitable amounts of 1° Ab and Revolune Connector. The following table shows an example calculation for staining 10 slides (using 2000 µL working solution), for various concentrations:

Primary antibody target	Primary Ab labelling reagent	A: Stock conc. 1° Ab [mg/mL] **(A)**	Working conc. 1° Ab [µg/mL] **(B)**	Working solution volume [mL] **(C)**	PBS	µL needed of 1° Ab **(D)**	µL needed of Revolune Connector **(E)**
Target 1	Revolune Connector 488	1.0	1.0	2	10 µL	2 µL	10 µL
Target 2	Revolune Connector 555	1.0	1.5	2	10 µL	3 µL	15 µL
Target 3	Revolune Connector 647	0.5	0.5	2	10 µL	2 µL	5 µL
Target 4	Revolune Connector 750	0.5	1.0	2	10 µL	4 µL	10 µL

The following formula and table may be used as template to calculate the amounts for your assay:

$D = (B \div A) \times C$

$E = B \times C \times 5 \frac{\mu L}{\mu g}$

Primary antibody target	Primary Ab labelling reagent	Stock conc. 1° Ab [mg/mL] **(A)**	Working conc. 1° Ab [µg/mL] **(B)**	Working solution volume [mL] **(C)**	PBS	µL needed of 1° Ab **(D)**	µL needed of Revolune Connector **(E)**
	Revolune Connector 488				10 µL		
	Revolune Connector 555				10 µL		
	Revolune Connector 647				10 µL		
	Revolune Connector 750				10 µL		

Mix PBS, 1° Ab and Revolune Connectors in four separate reaction tubes gently by flicking the tube, spin down the vials briefly and incubate for 30 min at room temperature (RT). The 1° Ab are now ready for the amplification step.

Part 2: Manual staining protocol

The volumes are calculated based on a working solution of 2 mL, i.e. 10 slides. If less slides are stained, this can be adjusted accordingly.

Step	Procedure	Done tick
1	Prepare the tissue section with Dewax and heat-induced epitope retrieval (HIER) based on established lab procedures. After this, the staining procedure takes approx. 6 hours.	☐
2	Draw a barrier around the tissue with an hydrophobic barrier PAP pen.	☐
3	For wash steps, prepare three staining jars with PBS-T wash buffer.	☐
4	Wash tissue slides three times by incubating them for 15 seconds in each prepared staining jar.	☐
5	Remove the last wash buffer by blotting the slide edges on a paper towel.	☐
6	As blocking step, add 180 µL antibody diluent (1% BSA in PBS-T, pH 7.4) and incubate for 15 min at room temperature (RT). Ideally, on a rotary shaker at RPM that allows gentle mixing of reagents.	☐
7	Meanwhile, generate the Ab cocktail by mixing the 1° Ab-Connector complexes (from Part 1) with antibody diluent (1% BSA in PBS-T, pH 7.4) for the calculated working solution (part 1, **C**), e.g. 2 mL. *Adjust as necessary for the number of slides to be stained.*	☐
8	Remove the dilution buffer from step 6 by blotting edges on a paper towel.	☐
9	Add 180 µL of the Ab cocktail (step 7) to each slide and incubate for 60 min at RT. Ideally, on a rotary shaker at RPM that allows gentle mixing of reagents.	☐
10	Remove the Ab cocktail and wash tissue slides three times by incubating them for 1 min in each prepared staining jar with PBS-T.	☐
11	Meanwhile, mix 1 mL Revolune Amplifier A and 1 mL Revolune Amplifier B in a reaction tube to generate the amplification solution 1. *Adjust as necessary for the number of slides to be stained, the ratio of the amplifiers is 1:1.*	☐
12	Remove the last wash buffer (step 10) by blotting the slide edges on a paper towel.	☐
13	Add 180 µL of amplification solution 1 (step 11) and incubate for 90 min at RT. Ideally, on a rotary shaker at RPM that allows gentle mixing of reagents.	☐
14	Remove the amplification solution 1 and wash tissue slides three times by incubating them for 1 min in each prepared staining jar with PBS-T.	☐
15	Mix 1 mL Revolune Amplifier C and 1 mL Revolune Amplifier D in a reaction tube to generate the amplification solution 2. *Adjust as necessary for the number of slides to be stained, the ratio of the amplifiers is 1:1.*	☐
16	Remove the last wash buffer (step 14) by blotting the slide edges on a paper towel.	☐
17	Add 180 µL of amplification solution 2 (step 15) and incubate for 90 min at RT. Ideally, on a rotary shaker at RPM that allows gentle mixing of reagents.	☐
18	Remove the amplification solution 2 and wash tissue slides three times by incubating them for 1 min in each prepared staining jar with PBS-T.	☐

19	Prepare DAPI counterstain solution, diluted in PBS-T (DO NOT use water or other diluents). We recommend using DAPI at 1 µg/mL. If the stock solution is 1 mg/mL, dilute 2 µL DAPI stock solution in 2 mL PBS-T.	☐
20	Remove the last wash buffer (step 18) by blotting the slide edges on a paper towel.	☐
21	Add 180 µL of DAPI counterstain solution (step 19) and incubate for 10 min at RT. Ideally, on a rotary shaker at RPM that allows gentle mixing of reagents.	☐
22	Wash tissue slides three times by incubating them for 1 min in each prepared staining jar with PBS-T.	☐
23	Prepare a fresh staining jar with PBS-T and incubate the slides for 1 min.	☐
24	Remove any excess liquid from the specimen by tapping the slide on to a clean tissue. **Do not wash the slide with water.**	☐
25	Place a drop or suitable amount ProLong Gold (e.g. 50 µL) onto a clean coverslip and carefully lower it onto the specimen. *Note: Other mounting media may lead to rapid degradation of the signal.*	☐
26	Allow the mounted slide to cure on a flat surface in the dark at room temperature. Curing time may vary from a couple of hours to overnight	☐
27	Image the samples as soon as possible after curing for best fluorescent signal.	☐
28	Store the samples at 4°C to best preserve the signal. It is stable for at least 1 week. Storing at room temperature is not recommended.	☐